# Epigenetic Changes in the Hypothalamic Proopiomelanocortin and Glucocorticoid Receptor Genes in the Ovine Fetus after Periconceptional Undernutrition

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Maternal food restriction is associated with the development of obesity in offspring. This study examined how maternal undernutrition in sheep affects the fetal hypothalamic glucocorticoid receptor (GR) and the appetite-regulating neuropeptides, proopiomelanocortin (POMC) and neuropeptide Y, which it regulates. In fetuses from ewes undernourished from −60 to +30 d around conception, there was increased histone H3K9 acetylation (1.63-fold) and marked hypomethylation (62% decrease) of the *POMC* gene promoter but no change in POMC expression. In the same group, acetylation of histone H3K9 associated with the hypothalamic *GR* gene was increased 1.60-fold and the GR promoter region was hypomethylated (53% decrease). In addition, there was a 4.7-fold increase in hypothalamic GR expression but no change in methylation of *GR* gene expression in the anterior pituitary or hippocampus. Interestingly, hypomethylation of both POMC and GR promoter markers in fetal hypothalami was also identified after maternal undernutrition from −60 to 0 d and −2 to +30 d. In comparison, the *Oct4* gene, was hypermethylated in both control and underfed groups. Periconceptional undernutrition is therefore associated with marked epigenetic changes in hypothalamic genes. Increase in GR expression in the undernourished group may contribute to fetal programming of a predisposition to obesity, via altered GR regulation of POMC and neuropeptide Y. These epigenetic changes in GR and POMC in the hypothalamus may also predispose the offspring to altered regulation of food intake, energy expenditure, and glucose homeostasis later in life. **(*Endocrinology* 151: 3652–3664, 2010)**

There is growing concern about the marked rise in incidence of patients with metabolic syndrome, which affects up to 25% of the population in the United States (1). At the core of the problem is the association between obesity and insulin resistance, which leads to increased risk of developing cardiovascular disease and diabetes (2).

There is considerable evidence to suggest that maternal undernutrition impacts on fetal development, leading to adult obesity (3–6). This is relevant not only for areas of the world where famine exists but also for developed countries where women of child-bearing age are encouraged to diet. It is thought that programming of the fetus generates changes that allow it adaption to decreased nutrition, which therefore are an advantage to a fetus born into an environment with limited access to food. This is described as the "thrifty phenotype" (7). However, subsequent increases in nutritional availability lead to increased risk of developing metabolic syndrome.

Mechanisms associated with the programming of metabolic syndrome are unclear, but alterations in the fetal hy-

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Abbreviations: ARC, Arcuate nucleus; ChIP, chromatin immunoprecipitation; GR, glucocorticoid receptor; H3K9Ac, acetylation of histone H3; HPA, hypothalamic-pituitary-adrenal; HSD, honestly significant difference; NPY, neuropeptide Y; POMC, proopiomelanocortin; UCSC, University of California, Santa Cruz.

pothalamic-pituitary-adrenal (HPA) axis (8, 9) have been implicated (9, 10). Glucocorticoids have well-defined direct negative feedback on the hypothalamus and pituitary (11) and also act on the hippocampus, which expresses high levels of glucocorticoid receptors (GRs) in both adult and fetal tissues and is a target for fetal programming of glucocorticoid responses (12–14). Periconceptional undernutrition in sheep accelerates maturation of the fetal HPA axis in late gestation, associated with elevated fetal baseline cortisol concentrations (15). Approximately half of these ewes deliver early, suggesting a potential link between periconceptional maternal nutrition and preterm birth (16). Therefore, programming of the HPA axis may also impact on mechanisms that predispose to metabolic syndrome in adult offspring.

However, it is likely that effects of maternal undernutrition manifest at the level of the fetal hypothalamus given that energy homeostasis is tightly controlled in the hypothalamus to cope with fluctuations in energy consumption and expenditure. In particular, maternal undernutrition could affect the arcuate nucleus (ARC) anorexigenic neurons, which express proopiomelanocortin (POMC) and the orexigenic neurons, which express neuropeptide Y (NPY) (17). POMC plays a central role in energy homeostasis (18) as evidenced by POMC knockout mice that develop obesity (19) and enhanced responsiveness to high-fat feeding (20). POMC mutations have also been associated with obesity in humans (21, 22). In addition, hypothalamic POMC has been shown to regulate glucose homeostasis directly, and this function is impaired in obesity (23).

There is evidence, from rodent studies, that maternal perinatal undernutrition disturbs the hypothalamic POMC anorexigenic circuit in newborn pups (24) and in adult offspring, where it is associated with changes in the regulation of food intake, body weight, and energy metabolism (25). Also, rats exposed to prenatal undernutrition and postnatal high-fat diet exhibited increased food intake and fat mass, which correlated with an increase in hypothalamic POMC (26).

In sheep, fetal effects of midgestation nutrient restriction showed no change in fetal hypothalamic POMC expression at birth but a significant increase in expression at 1 yr of age when exposed to an obesogenic environment, thus suggesting that the expression of the *POMC* gene was programmed despite no initial change and that different windows of sensitivity may exist in different animal models (27). There is, also, evidence that periconceptional undernutrition in sheep results in glucose intolerance in postpubertal offspring (28).

It is not clear how maternal undernutrition would affect fetal hypothalamic feeding centers in a way that would lead to inappropriate responses to high-fat diets in adult offspring. However, epigenetic programming of candidate genes in hypothalamic neurons seems a likely mechanism. Patterns of genomic DNA methylation are established during early embryonic development and then faithfully maintained through life by DNA methyltransferases (29–31). Acetylation of histones, including histone H3 (H3K9Ac) opens compact chromatin structures and allows transcription (32). The association of epigenetic changes with changes in mRNA expression is not necessarily acute; for instance, methylation of the GR exon 1 promoter region has been associated with long-term changes in gene expression due to changes in transcription factor binding (33). Therefore, a change in genomic DNA methylation can only result in a change in transcription when the transcription factor in question is present, whether this is acute or chronic depends on the transcription factor.

Changes in histone acetylation have been associated with the transcriptional regulation of POMC by glucocorticoids (34). Recently, a rat model of neonatal overfeeding has shown hypermethylation of the hypothalamic POMC promoter compared with constant hypomethylation of the *NPY* gene, implicating plasticity in the POMC promoter as a critical "set-point" for body weight regulation (35). However, nothing is currently known about comparable changes after undernutrition.

The GR has been implicated in programming with methylation of the GR exon 1 promoter region being associated with long-term changes in gene expression (30). Fetal exposure to maternal glucocorticoids also affects the HPA axis (36, 37). There are well-established causal relationships between the epigenetic state, GR expression, and the effects of maternal care on stress responses in rodent offspring (38). Undernutrition is known to decrease hypothalamic GR expression in adult rats (39, 40). Although glucocorticoids are key regulators of hypothalamic neuropeptides (41, 42), the potential role of epigenetic programming of the GR on regulation of energy homeostasis is unclear.

We and others have shown that hypothalamic neuropeptides are altered in obese Zucker rats (43–45), suggesting that neurons in this region may be an important target for epigenetic changes associated with maternal undernutrition. It has also been shown that sheep whose mothers were subjected to periconceptional undernutrition are heavier than controls at 10 months of age (28). Therefore, in this study, we examined whether epigenetic changes in POMC and GR were present in the hypothalami of late gestation fetuses from undernourished mothers using a sheep paradigm, chosen because of the similarity with the human fetal HPA axis and placental function

(46). We found that both *POMC* and *GR* genes are associated with changes in histone acetylation and promoter methylation. In addition, GR expression is already increased in fetal hypothalamic feeding centers. Perhaps more importantly, the hypomethylation of these POMC and GR promoter markers could influence POMC regulation and glucocorticoid effects on other hypothalamic energy regulating pathways. Early life epigenetic regulation of these neuropeptides by maternal nutritional status may therefore have implications for the glucose homeostasis, food intake, and energy balance of the adult offspring.

## Materials and Methods

### Animal management, nutritional manipulation, and surgery

Experiments were approved by the Animal Ethics Committee at the University of Auckland. Multiparous 4–5 yr old Romney ewes were acclimatized to a concentrate feed (CamTech, Cambridge, New Zealand) (47), then randomly divided into four groups: controls (*ad libitum* feeds at 3–4% of body weight per day); undernutrition from 60 d before until mating (−60 to 0), from 2 d before mating until 30 d after (−2 to +30), or from 60 d before until 30 d after mating (−60 to +30). Undernutrition comprised a 2-d fast, and then concentrates were individually adjusted to achieve and maintain 10–15% body weight reduction (47). Food intake was initially 1–2% of body weight per day, increasing to approximately 80% of controls (47). Ewes were fed *ad libitum* when not undernourished. After ultrasound scanning at 55 d, only singleton-bearing ewes were retained. Fetal and maternal catheterization was carried out at d 127 of gestation. The fetus was exposed through a midline incision in the maternal abdomen, and polyvinyl catheters were inserted into the tarsal artery and vein of both fetal hind limbs. Maternal femoral artery and vein catheters were also inserted. Fetal arterial blood samples (4 ml) were collected into chilled heparinized tubes twice daily at 0800 and 2000 h from d 131 to 135 of gestation for later plasma analysis. To collect fetal tissue, ewes were killed on gestational d 131 and 135 by a lethal iv dose of injectable pentobarbitone. Fetal hypothalami and pituitaries were dissected and immediately frozen until assay (47).

### Bioinformatic analysis of sheep genome

The human genome sequence (build GRCh37/hg19) and bovine genome sequence (build Baylor 4.0/bos Tau4) were used as a base to map the promoter regions of the *POMC*, *GR*, and *Oct4* genes 12 kb downstream of the ATG translational start site. CpG content was examined by sequence analysis and by GC content in a 5-bp widow [University of California, Santa Cruz (UCSC), please see http://genome.ucsc.edu/]. Areas of highly conserved mammalian sequence homology were identified from the UCSC website using multiple alignments of five vertebrate species (cow, dog, human, mouse, and platypus) with a measure of evolutionary conservation, based on a phylogenetic hidden Markov model, phastCons (48). Primers designed from the consensus sequence of these conserved homologous regions were used to amplify ovine genomic DNA. Successfully amplified ovine genomic DNA was sequenced and homology with the conserved consensus sequence was analyzed using ClustalW 2.0 (European Molecular Biology Laboratory-European Bioinformatics Institute).

Primers used to generate the amplicons were as follows: GR, 5′-TTTGGAGGGACTGTGGTCC-3′, 5′-AGCAGGAGGTGGCAGGCC-3′, size: 230 bp; for POMC, 5′-ACCCTCAGAGGTGAGAAGCT-3′, 5′- GGAAGGAGACCGGAGCCG-3′, size: 160 bp; for Oct4, 5′- CCTGGATGAGCTTCCAAGG-3′, 5′-CCTCGGAGTTGCTCTCCCAC-3′, size: 223 bp.

### Hormone and peptide assays

The presence of POMC in fetal plasma samples was measured by ELISA, based on the immunoradiometric assay described previously (49) and shown to work in sheep (50). The assay does not cross-react with the bioactive peptide derivatives of POMC, ACTH (<3.6%) or α-MSH (<2.2%), but does recognize both POMC and pro-ACTH (100%) (51). The limit of sensitivity of the POMC ELISA was 10 pmol/liter.

Plasma ACTH was measured using a commercial RIA (Diasorin, Stillwater, MN) previously validated for use in the sheep (52). Intra- and interassay coefficients of variation for the ACTH RIA were 11.8 and 5.6%.

Cortisol was measured using mass spectrometry as previously described (53).

### Quantitative analysis of mRNA expression

RNA from tissue was extracted with RNeasy kit (QIAGEN, Valencia, CA). Quantitative expression analysis of mRNA was undertaken using the Quantigene II system (Panomics, Inc., Freemont, CA) (54). Quanti-Gene assays were performed according to the manufacturer's protocol. Briefly, 2 μg of total RNA in 10 μl of ribonuclease-free water was loaded into each well of a 96-well plate and incubated with sheep target probes [probes synthesized by Panomics, Inc. from the following accession numbers: POMC, NM001009266; NPY, NM001009452; GR, NM001114186; or glyceraldehyde-3-phosphate dehydrogenase, AF030943], in conjunction with dendrimer DNA amplifier and labeled probes at 56 C overnight. After washes, chemiluminescent substrate was added to the wells and incubated at 56 C for 30 min, then read on a Mithras luminometer (Berthold, Pforzheim, Germany).

### Methylation-specific PCR analysis

Tissue specific methylated genomic DNA was enriched from total genomic DNA using the MethylCollector kit (ActiveMotif, Carlsbad, CA). Briefly, CpG methylated DNA was digested with MseI restriction endonuclease (New England Biolabs, Hitchin, UK), bound specifically to His-tagged recombinant methyl-CpG-binding domain protein 2b protein, captured with nickel-coated magnetic beads, and subsequent wash steps were performed with a stringent high-salt buffer to remove fragments with little or no methylation. The methylated DNA was then eluted from the beads. PCR was performed on input MseI digested DNA and compared with methylation-enriched DNA to establish relative differences in methylation state. PCR was performed in a final volume of 50 μl, containing 10 pmol of each primer, 200 μmol/liter of each dNTP, 2.5 U of *Taq* polymerase (QIAGEN), and 3 μl of DNA template. The initial denaturation (97 C, 5 min) was followed by 30 cycles of 1 min at 95 C, 1 min at 58 C, 1 min at 72 C, and a final extension step at 72 C for 10 min. PCR band intensity for POMC, GR, and Oct4 amplicons

was quantified using ImageJ software (developed by Wayne Rasband; National Institutes of Health, Bethesda, MD).

### Chromatin immunoprecipitation (ChIP)

ChIP studies were performed using the Imprint ChIP kit (Sigma, St. Louis, MO). Homogenized hypothalamic tissue (20 mg) was cross-linked with 1% formaldehyde, the chromatin isolated, incubated with cell lysis buffer, and digested with micrococcal nuclease (2 U/ml; Sigma) to generate fragments of genomic DNA with an average size of 500 bp. Equal amounts of digested chromatin were immunoprecipitated with 1 $\mu$g of each of the following antibodies: mouse IgG (M8695; Sigma), RNA polymerase II (R1530; Sigma), or Rabbit polyclonal histone H3 acetylK9 (H3K9Ac; Abgene, Rochester, NY; ab12178-50). The chromatin was washed, and the cross-links were hydrolyzed. The DNA was then used for PCR analysis using the same amplicons and conditions for GR and POMC as used for the methylation PCR (described above). PCR band intensity was quantified using ImageJ software.

### Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using the unpaired Student's *t* test, repeated measures ANOVA or one-way ANOVA, and a Tukey honestly significant difference (HSD) *post hoc* test as appropriate using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

## Results

### Identification of POMC and GR amplicons for ChIP and methylation analysis of ovine genomic DNA

The choice of DNA amplicons in this study was limited by the lack of available sheep genomic sequence. However, we were able to locate amplicon sequences based on conservation of sequence homology with mammalian genomes. The cow genome in particular is highly homologous to the sheep and has been used to generate virtual models of the sheep genome to aid in the creation of a genomic "scaffold" (55).

Homology analysis of the *POMC* gene region identified one candidate region (Fig. 1A) of highly conserved sequence (Fig. 1B) that was proximal to CpG islands. The same approach was used to screen the *GR* and *Oct4* gene regions (the latter used as a control), and similarly, one candidate region was identified in each gene (Fig. 1C) (data not shown) with highly conserved sequence that was within a CpG island (Fig. 1D) (data not shown). Primers were designed using the consensus sequence and used to amplify ovine genomic DNA, the ovine sequence was subsequently confirmed by sequencing (these sequence data have been submitted to the GenBank database under accession nos. GR, HM118850; POMC, HM118849; and Oct4, HM118848).

The marker of the ovine POMC promoter region used in this study started 6.5 kb from the 5′ end of exon 1 (relative to human POMC sequence), where there is an associated CpG island studied previously in human cells and associated with expression of the *POMC* gene (56, 57). This sequence was CpG rich and located 2.5 kb downstream from regions homologous to the conserved hypothalamic enhancer regions (58, 59) and their associated CpG islands (Fig. 1A).

The *GR* gene promoter marker identified was located 5 kb upstream of the translational start site in exon 2 (Fig. 1C) within the 5′ region of the CpG island associated with the transcriptional start sites of the exon 1 region as mapped by homology to the human genome (Fig. 1D). This amplicon was CpG rich and proximal to glucocorticoid regulatory regions defined in the human genome (60).

The *Oct4* gene has been shown to be a hypermethylated pluripotency gene in humans (61) and was selected for this study to act as a control for methylation. A CpG-rich region is present in the *Oct4* gene over the translational start site that shows conserved homology between human, cow, dog, and platypus genomes (data not shown). A sheep amplicon was selected within this Oct4 CpG island 1 kb downstream of the translational start identified by conserved homology.

### Baseline HPA axis activity is not changed in fetuses from mothers with periconceptional undernutrition

There was no significant difference in fetal plasma POMC concentrations between fetuses from the maternal underfed and control groups over 96 h between d 131 and 135 of pregnancy (Fig. 2A). This time period was selected because it was before the cortisol surge that correlates with parturition (62). Similarly, there was no difference in total amounts of fetal plasma POMC, ACTH, and cortisol present over this period (area under the curve analysis) (Fig. 2B).

Immunohistochemistry demonstrated POMC expression in the anterior pituitary and intermediate lobe of fetal tissue from d 135 (data not shown). Methylation and mRNA expression of the POMC promoter marker was assessed in fetal pituitary tissue for comparison with hypothalamic data. Methylation of the POMC promoter region was not altered in anterior pituitary fetal tissue from underfed mothers compared with controls (Fig. 2C), and POMC mRNA expression was unchanged between the groups (Fig. 2D). POMC expression in the pituitary was up to 200-fold greater than observed in the hypothalamus as expected.

### H3K9Ac associated with the hypothalamic POMC promoter in fetuses from mothers with periconceptional undernutrition

Hypothalami from d 135 fetal sheep expressed POMC, NPY, and GR protein (data not shown), markers of a functional nutritional regulatory pathway in the hypo-

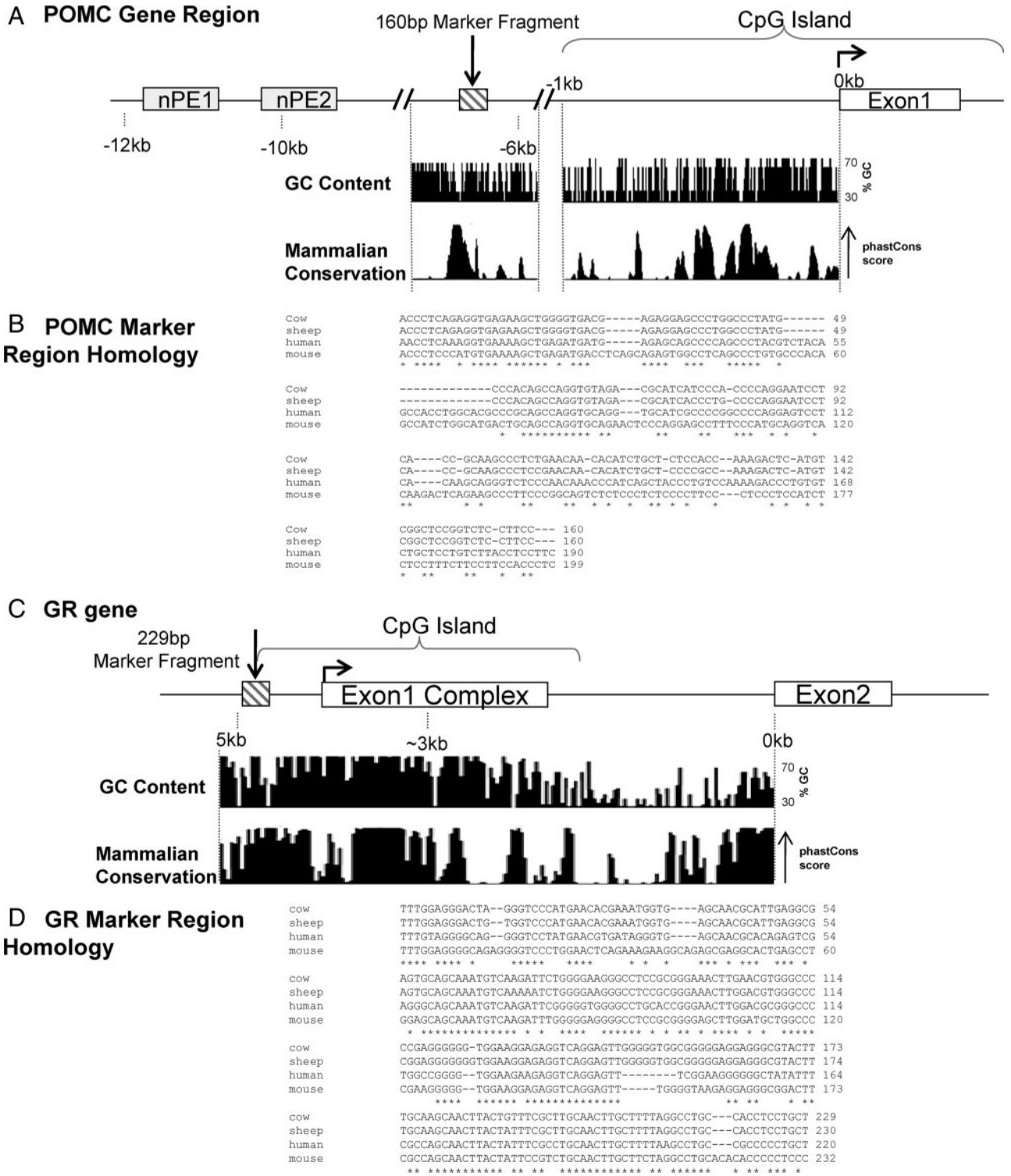


**FIG. 1.** POMC and GR gene region screening to identify highly conserved, CpG-rich regions. A, The *POMC* gene region with GC content and mammalian sequence homology mapped from the bovine genome (UCSC database). B, Direct comparison of the POMC amplicon marker region in cow, sheep, mouse, and human sequences. C, The *GR* gene region with GC content and mammalian sequence homology mapped from the bovine genome (UCSC database). D, Direct comparison of the GR amplicon marker region in cow, sheep, mouse, and human sequences. Sequence homology mapping was performed using the phastCons software (48). *Striped box* indicates promoter marker region. nPE, Neuronal POMC enhancer.

thalamus. POMC expression was localized, by immunohistochemistry, mainly in the ARC of the fetal sheep ventral hypothalamus (data not shown).

H3K9Ac on the POMC promoter marker was examined using ChIP (Fig. 3A). Chromatin from the ventral hypothalami of d 131 fetuses from groups of control ewes

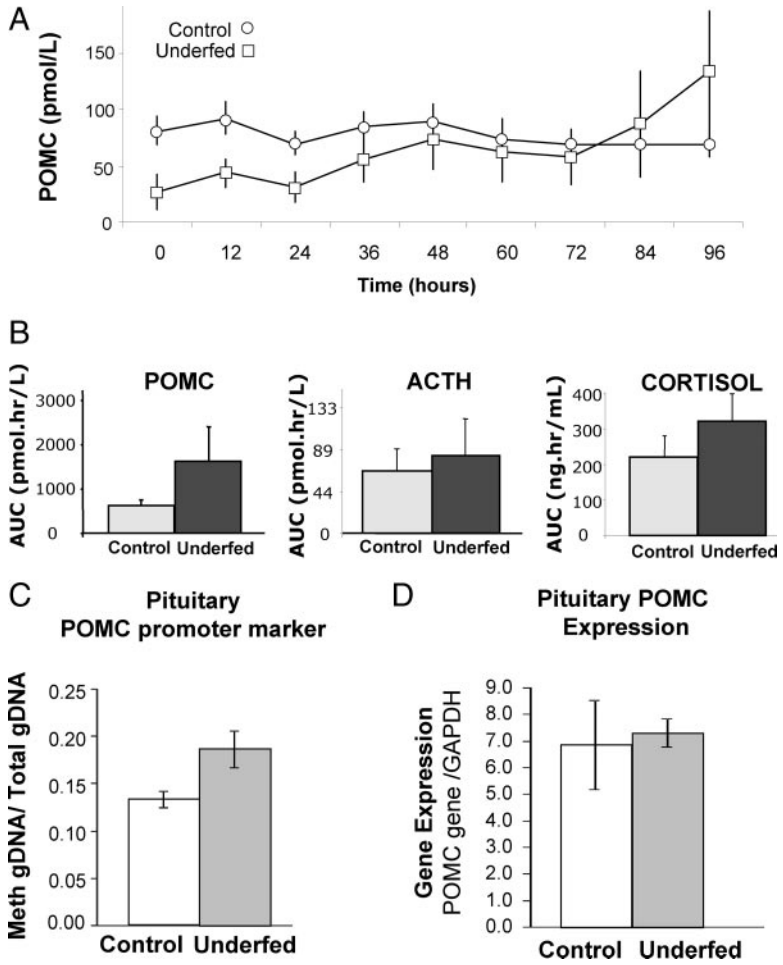


**FIG. 2.** HPA axis activity in fetal sheep from control ewes or ewes subjected to periconceptional undernutrition (underfed from 60 d before conception to 30 d after conception). A, Plasma POMC concentration over 96 h starting at d 131 of pregnancy (control group, n = 8; undernutrition group, n = 6). B, Area under the curve (AUC) analysis over 96 h from d 131 of pregnancy for POMC (pmol/h · liter), ACTH (pmol/h · liter), and cortisol (ng/h · ml). C, Pituitary tissue samples were obtained from the fetuses for promoter region methylation analysis of the *POMC* gene promoter marker amplicon in the pituitary (control, n = 6; underfed, n = 6). D, Expression levels of the *POMC* gene in total RNA purified from pituitary samples (control, n = 6; underfed, n = 6).

and groups of ewes subjected to periconceptional undernutrition from −60 to +30 d relative to conception was immunoprecipitated with a polyclonal antibody to histone H3 acetyl-K9 (Fig. 3B). There was a 1.63-fold increase of H3K9Ac in ventral hypothalami from the fetuses of the underfed group compared with the control group ($P < 0.001$).

RNA Polymerase II was used as a positive control identifying binding to the POMC marker region when it is associated with the transcriptional machinery (Fig. 3A). This occurs via folding of the promoter to bring the marker region in close proximity to the transcription start site. This mechanism has been shown previously for POMC, when RNA Polymerase II was immunoprecipitated with a promoter region fragment approximately 400 bp upstream of the transcriptional start site (34). The association of promoter elements distant to the transcriptional start site have also been shown to effectively immunoprecipitate with RNA Polymerase II in a genomic screen (63).

### Methylation of the hypothalamic *POMC* gene in fetuses from undernourished ewes

Initially, whole hypothalami were taken from fetuses of a group of normally nourished and underfed animals at d 135 of pregnancy. Analysis of the POMC marker region in the whole hypothalami revealed a 64% decrease in methylation in the fetal hypothalami from the underfed group compared with the controls ($P < 0.01$) (Fig. 3C).

POMC from tissue restricted mainly to the ARC was also assessed in a group of animals subjected to periconceptional undernutrition (from −60 to +30 d relative to conception) but killed at d 131 of pregnancy. Genomic DNA from ventral hypothalamic slices containing the third ventricle revealed a 62% decrease in POMC marker methylation in the fetal hypothalami from the underfed group compared with the control group ($P < 0.001$) (Fig. 3D).

### Quantitative analysis of *POMC* gene expression in the hypothalamus of fetuses from undernourished ewes

There was no difference in expression of POMC in the maternally underfed group compared with the control group in the whole hypothalami or the ventral hypothalami (from d 135 and 131 fetuses, respectively) (Fig. 4). NPY expression was not altered in either whole hypothalami (Fig. 4A) or the arcuate-enriched ventral region (Fig. 4B).

### H3K9Ac on the hypothalamic *GR* gene in fetuses from undernourished ewes

Because glucocorticoids are known to play a role in appetite regulation, we assessed the effect of periconceptional undernutrition on the hypothalamic *GR* gene. In rats, a CpG island is found approximately 2.5-kb 5′ of exon 2 associated with the complex of different exon 1 regions. Hypomethylation of this region has been associated with increased GR expression (64). The *GR* gene exon 1 region is well characterized in humans and rats and as the start site for GR tran-

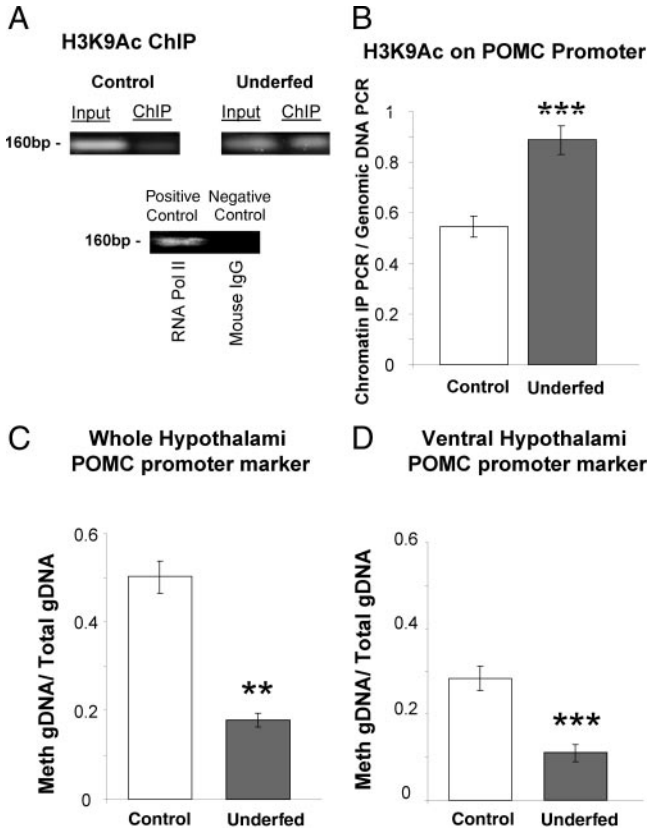


**FIG. 3.** Epigenetic changes associated with the *POMC* gene in the fetal hypothalamus. Fetal hypothalamic tissue samples were obtained from normal and underfed maternal sheep (underfed from 60 d before to 30 d after conception). A, Genomic DNA (gDNA) was purified from the tissue. DNA immunoprecipitated with a histone H3K9 acetylation antibody was then used for PCR to detect the presence of the *POMC* gene promoter marker. An antibody to RNA Polymerase II (RNA Pol II) was used as a positive control and mouse IgG as a negative control. B, Ratio of PCR signal from H3K9Ac immunoprecipitated DNA to total genomic DNA for DNA purified from gestational age d 131 ventral hypothalamic sections (control, n = 9; underfed, n = 11). C, Ratio of *POMC* gene promoter marker PCR signal from methylated genomic DNA to total genomic DNA for whole hypothalami at gestational age d 135 (control, n = 3; underfed, n = 3). D, Ratio of *POMC* gene promoter marker PCR signal from methylated genomic DNA to total genomic DNA for ventral hypothalamic sections at gestational age d 131 (control, n = 9; underfed, n = 11). **, $P < 0.01$; ***, $P < 0.001$.

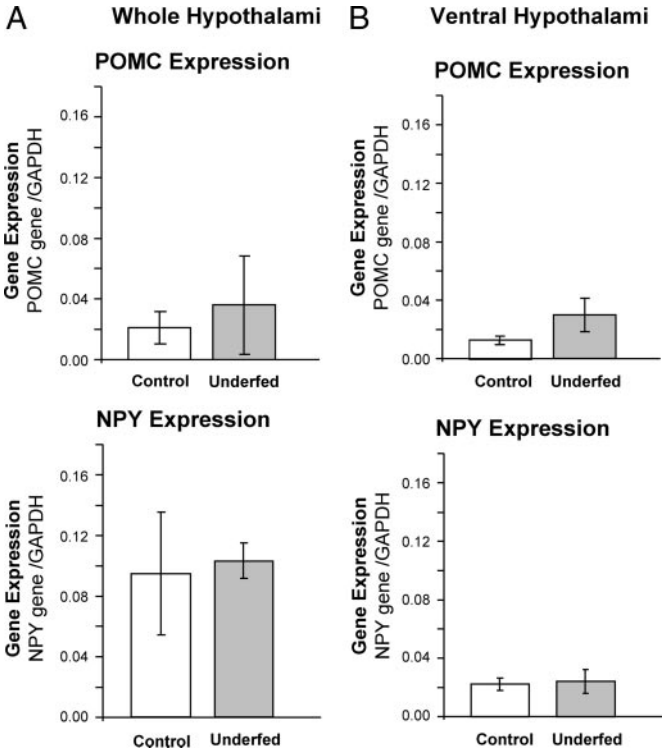


**FIG. 4.** Expression of the *POMC* gene in the fetal hypothalamus. Fetal hypothalamic tissue samples were obtained from control and underfed (from 60 d before conception to 30 d after conception). Total RNA was used to quantify expression levels of *POMC* and *NPY* genes. POMC and NPY expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A, Expression levels of POMC and NPY in total RNA purified from whole hypothalami at gestational d 135 (control, n = 3; underfed, n = 3). B, Expression levels of POMC and NPY in total RNA purified from ventral hypothalamic sections at gestational age d 131 enriched for ARC (control, n = 9; underfed, n = 11).

scription (65). The sheep GR promoter region marker identified in this study was located in a similar CpG region and was used to examine the presence of H3K9Ac, as a marker of transcriptional activity (Fig. 1C).

To investigate *GR* gene promoter H3K9Ac status, homogenized tissue was used from the same ventral hypothalamic samples taken for the POMC analysis (Fig. 5A). Genomic DNA fragments pulled down with the immunoprecipitation were used as PCR template for the 229-bp sheep GR promoter region marker (Fig. 1C). There was a 1.60-fold increase of H3K9Ac in ventral hypothalami from the fetuses of the underfed group compared with the normal group ($P < 0.001$) (Fig. 5B).

## Hypomethylation of the hypothalamic *GR* gene in fetuses from undernourished ewes

Analysis of the sheep GR promoter marker region in the whole hypothalami revealed a 40% decrease in methylation in underfed fetuses compared with controls ($P < 0.05$) (Fig. 6A). GR is ubiquitously expressed in the hypothalamus with a higher level of expression in the paraventricular nucleus, where it is involved with regulation of POMC and NPY (41). GR expression levels are also altered in the ventral hypothalamus in food restricted rats (39). Therefore, we examined tissue from the ventral hypothalamus that contained mainly the ARC as previously used for POMC analysis. Genomic DNA was extracted from the ventral hypothalamic slice and enriched for methylated DNA. There was a 53% decrease in methylation in the fetal ventral hypothalami from the underfed group compared with the control group ($P < 0.05$) (Fig. 6B).

In comparison, methylation of this GR promoter region was shown to be unaltered in fetal pituitary and hippocampal tissue in the group from underfed mothers compared with controls (Fig. 6, C and D).

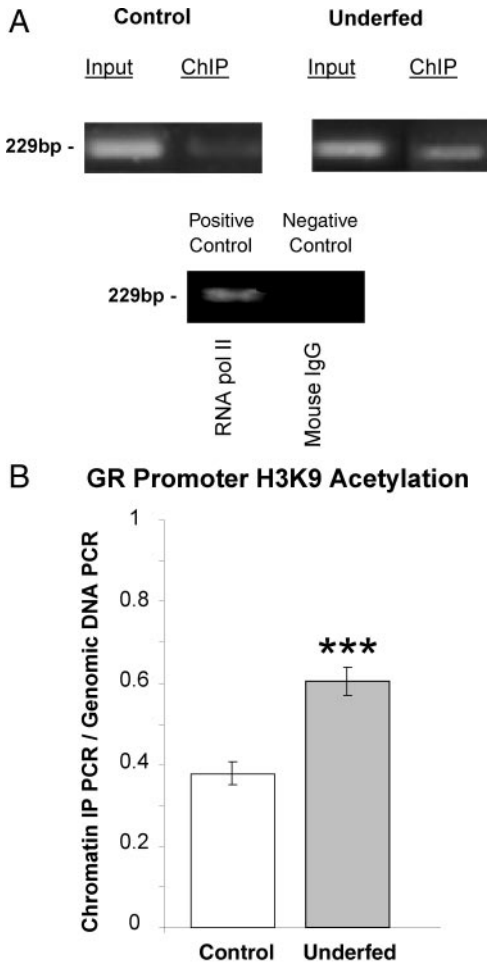


**FIG. 5.** Presence of H3K9Ac associated with the *GR* gene promoter in the fetal hypothalamus. B, Ratio of PCR signal from H3K9Ac immunoprecipitated DNA to total genomic DNA purified from ventral hypothalamic sections enriched for ARC (normal, n = 9; underfed, n = 11). ***, $P < 0.001$. Fetal hypothalamic tissue samples were obtained from normal and underfed maternal sheep (underfed from 60 d before conception to 30 d after conception) at d 131 of gestation. Genomic DNA was used for ChIP analysis with an antibody specific to H3K9Ac. A, PCR confirmed the presence of a 229-bp marker fragment of the *GR* gene promoter CpG island. RNA Pol II, RNA Polymerase II.

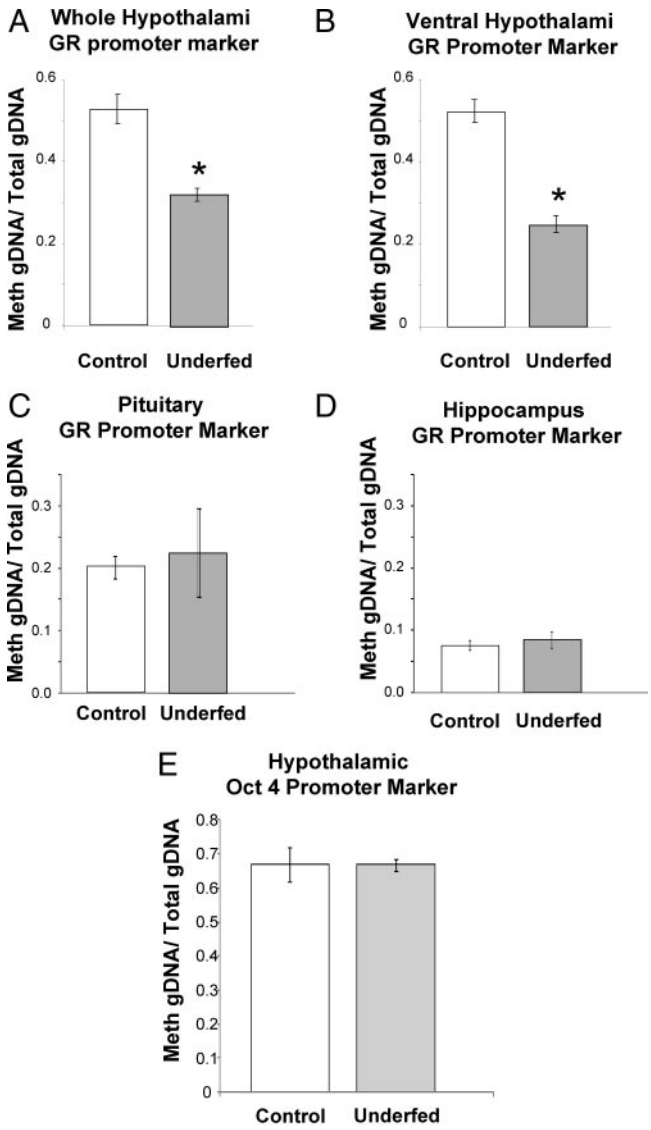


**FIG. 6.** Methylation of *GR* gene promoter region in the fetal hypothalamus. Hypothalamic tissue samples were obtained from control and underfed (from 60 d before conception to 30 d after conception) fetal sheep. A, Ratio of GR amplicon signal from methylated genomic DNA (Meth gDNA) to total genomic DNA for DNA purified from the whole hypothalami (control, n = 3; underfed, n = 3). B, Ratio of GR amplicon signal from methylated genomic DNA to total genomic DNA for DNA purified from ventral hypothalamic sections enriched for ARC (control, n = 9; underfed, n = 11). *, $P < 0.05$. C, Ratio of GR amplicon from methylated genomic DNA to total genomic DNA for DNA purified from pituitary samples at gestational age d 131 (control, n = 12; underfed, n = 12). D, Ratio of GR amplicon from methylated genomic DNA to total genomic DNA for DNA purified from hippocampal samples at gestational age d 131 (control, n = 12; underfed, n = 12). E, Ratio of Oct4 amplicon from methylated genomic DNA to total genomic DNA for DNA purified from ventral hypothalamic samples at gestational age d 131 (control, n = 4; underfed, n = 6).

The *Oct4* gene is hypermethylated across a range of human tissues (61). A sheep amplicon that marked the CpG island surrounding the translational start site of the *Oct4* gene was shown to be hypermethylated in sheep fetal hypothalamic tissue in both undernourished and control groups with no difference between groups (Fig. 6E).

### Quantitative analysis of *GR* gene expression in hypothalami of fetuses from undernourished ewes

In whole hypothalami, there was a 1.8-fold increase in the expression of the *GR* gene in the underfed compared with the control group ($P < 0.05$) (Fig. 7A), where we had previously shown no change in expression of the *NPY* gene (Fig. 4A). In the ventral hypothalami, there was a 4.7-fold increase in *GR* gene expression ($P < 0.05$) (Fig. 7B), where we had previously shown no change in *NPY* gene expression (Fig. 4B).

In pituitary and hippocampal tissue, fetal *GR* gene expression was unchanged between the fetuses from the maternal underfed group and the control group (Fig. 7C).

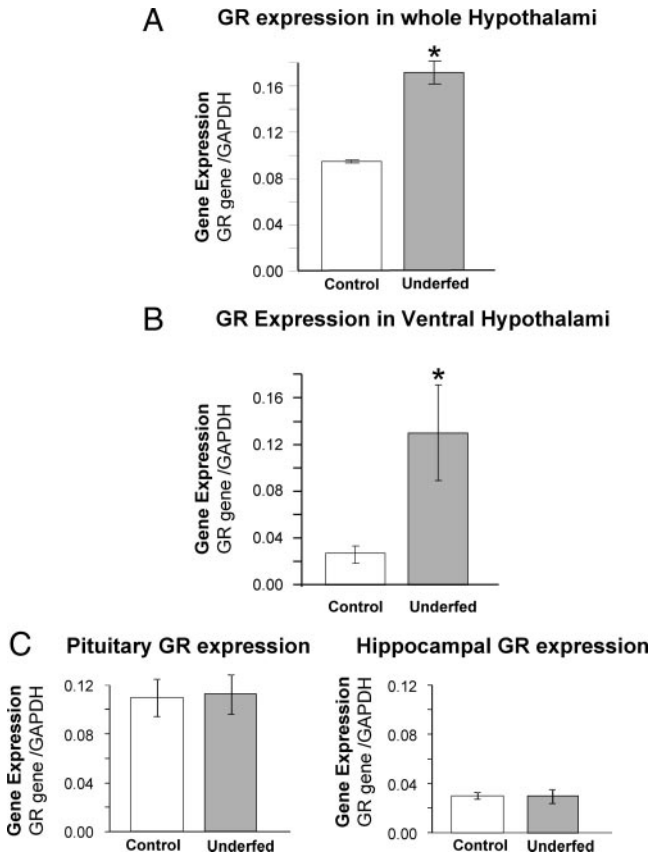


**FIG. 7.** Expression of *GR* gene in the fetal hypothalamus. Fetal hypothalamic tissue samples were obtained from control and underfed maternal sheep (from 60 d before conception to 30 d after conception); 2 $\mu$g of total RNA were used to quantify expression levels of GR. A, Expression levels of GR and NPY in total RNA purified from the whole hypothalami (control, n = 3; underfed, n = 3). B, Expression levels of GR in total RNA purified from ventral hypothalamic sections enriched for ARC (control, n = 9; underfed, n = 11). C, Expression levels of GR in pituitary and hippocampal tissue from fetal sheep at gestational d 131. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

### The effect of duration of undernutrition on hypothalamic *GR* and *POMC* genes

The investigation of epigenetic changes was extended to other periods of periconceptional undernutrition, *i.e.* from −60 d to conception (−60 to 0 d) or from conception to 30 d after (-2 to +30d). Methylation of both POMC and GR promoter markers was significantly reduced in all undernutrition groups compared with controls (Fig. 8A). POMC expression was not increased in any of the undernutrition groups compared with controls. However, there was a marked increase in the expression of the *GR* gene in the −60 to +30 d group (Fig. 8B). The expression of the *NPY* gene in the enriched hypothalami was not significantly different to controls (Fig. 8B).

## Discussion

This study has identified important epigenetic changes in *POMC* and *GR* genes in the fetal hypothalamus after periconceptional maternal undernutrition. Expression of GR mRNA is increased in fetal hypothalami collected 100 d after the periconceptional undernutrition of the mother ceased, and this is associated with promoter hypomethylation and increased H3K9 acetylation. In contrast, the *POMC* gene from late-gestation fetal hypothalami after maternal periconceptional undernutrition shows no change in expression of mRNA, although the *POMC* gene promoter region is hypomethylated and H3K9 acetylation levels are increased. This provides evidence for epigenetic changes that could act as a programming mechanism for the hypothalamic *POMC* and *GR* genes and predispose hypothalamic feeding centers to abnormal regulation later in life. Indeed, 10-month-old offspring from ewes undernourished using the same protocol were found to have abnormal glucose tolerance and increased body weight (28).

The hypomethylation of both *POMC* and *GR* genes occurs after maternal undernutrition irrespective of the developmental window during which the undernutrition occurred. The observation of similar levels of hypomethylation in both fetal *POMC* and *GR* genes across different periods of periconceptional undernutrition suggests that short time frames either side of conception are sufficient to alter regulatory patterns of fetal hypothalamic genes. This is consistent with observations of the differences in fetal glucose-insulin and HPA axis responses between these groups (47).

It would appear that an early nutritional insult alters the development of fetal hypothalamic appetite regulation centers to increase postnatal survival, assuming a continuation of the undernutrition. This suggests that an adaptive response by the fetus to undernutrition allows an increased chance of postnatal survival (7). However, if such changes persist in adulthood when food is abundant, a defect in normal appetite regulation may subsequently lead to overeating and obesity. This may explain several findings that animals undernourished prenatally are hyperphagic when given hypercaloric or high-fat diet postnatally, compared with control animals (31, 66). The current study has not addressed fetal weight and catch up growth, but in a previous study, using the same protocol, fetuses from undernourished mothers grew more slowly than well-nourished controls, but this was not reflected by size in late gestation (47).

Significant hypomethylation of the POMC promoter marker was observed in this study, which would suggest that the promoter region is made more accessible to regulation, but this would not necessarily lead to altered expression of the *POMC* gene at this prenatal stage. Hypomethylation of this marker may indicate hypomethylation across elements of the POMC promoter CpG island. This may therefore be

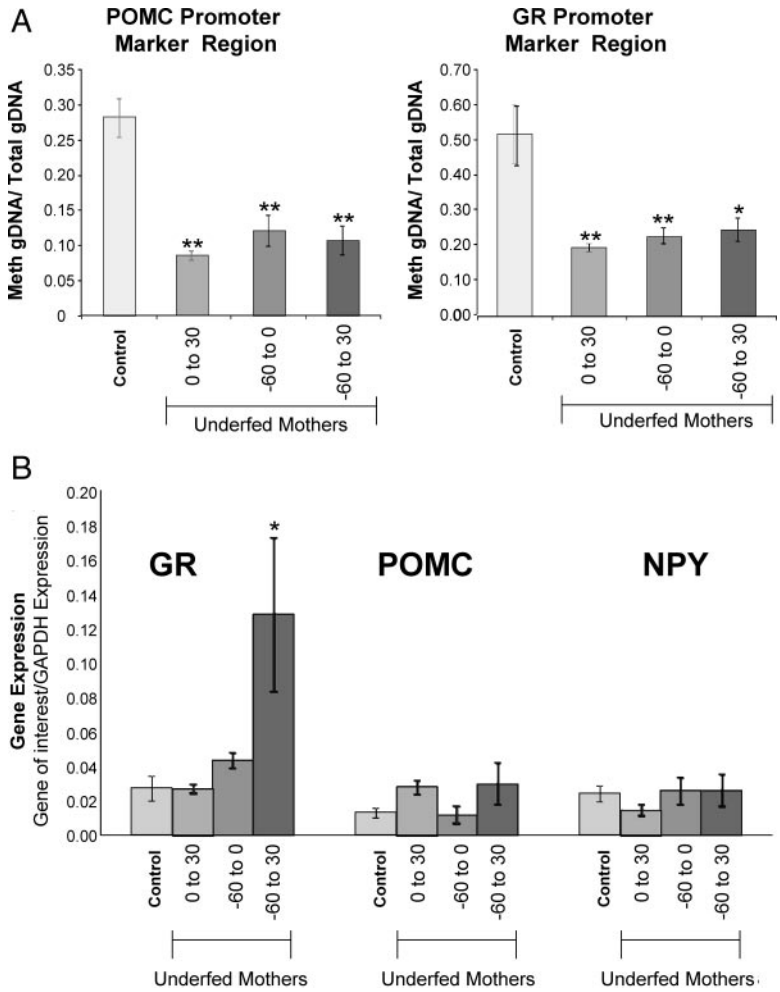


**FIG. 8.** The effect of different periods of periconceptional undernutrition on hypothalamic *GR*, *POMC*, and *NPY* methylation and gene expression. The −60 to +30 group (UN −60 to +30) was underfed from 60 d before conception to 30 d after conception. The −60 to 0 group (UN −60 to 0) were fed the same diet as the −60 to +30 group but were allowed to feed *ad libitum* from conception. The −2 to +30 group (UN −2 to +30) were fed the same diet for 30 d after conception. Fetal ventral hypothalamic tissue samples, enriched for the ARC, were obtained from all sample groups at d 131 of gestation. A, Methylation of GR and POMC promoter marker in ventral hypothalami from controls (n = 7), −60 to +30 (n = 9), −60 to 0 (n = 8), and −2 to +30 (n = 7) feeding regimens. The 229-bp marker of the *GR* gene and the 160-bp marker of the *POMC* gene promoter region CpG islands were used to compare the ratio of methylated with unmethylated DNA. One-way ANOVA with Tukey HSD *post hoc* test compared with control group. *, $P < 0.05$; ***, $P < 0.001$. B, Transcriptional expression of POMC, GR, and NPY in the ventral hypothalami from controls (n = 7), −60 to +30 (n = 11), −2 to +30 (n = 7), and −60 to 0 (n = 8) feeding regimens. One-way ANOVA with Tukey HSD *post hoc* test compared with all other groups. *, $P < 0.05$. Meth gDNA, Methylated genomic DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UN, Underfed.

associated with the changes in transcription factor binding identified by Newell-Price *et al.* (57) and also mark the region associated with cortisol-dependent demethylation identified by Mizoguchi *et al.* (66). Recently, hypomethylation of part of the proximal *POMC* gene promoter was associated with food restriction, in a rat model of maternal undernutrition, while equivalent regions in *CART* and *NPY* were not altered (67).

Silencing mechanisms associated with methylated genomic DNA can involve direct interference between a methylated CpG and the binding of a transcription factor, as has been shown with the interaction between the nerve growth factor inducible-A transcription factor and the *GR* gene (68, 69). Plagemann *et al.* (35) have examined the role of methylation in the regulation of hypothalamic POMC expression in a rat neonatal overfeeding model and demonstrated hypermethylation in SP1 binding regions. However, we were not able to examine these regions directly because there is no ovine genomic sequence currently available for the homologous regions. Highly conserved hypothalamic-specific POMC regulatory regions have been identified approximately 9 kb upstream of the start of exon 1 (Fig. 1A) (58, 59), and it is possible that the POMC promoter marker region used in this study acts as a marker of methylation changes within this region or in as yet undiscovered tissue specific regulatory regions.

Rats undernourished perinatally have decreased hypothalamic POMC expression with maternal undernutrition (24), and the long-term appetite-regulatory system of offspring is altered (25). In the current study, there was no significant difference in hypothalamic POMC expression between the maternally underfed and the control group, in line with previous work (26, 37). However, when these offspring were fed a high-fat diet, there were alterations in *POMC* gene expression (26). Delahaye *et al.* (24) measured POMC in postnatal life as opposed to fetal life, and therefore, it may be that in the current model, changes may be observed at an equivalent developmental postnatal age (24, 47). Caution must be observed with the comparison of data generated in rats with observations made in sheep and humans in that the hypothalamic neuroendocrine circuitry is established after birth in rodents and before birth in both sheep and humans, potentially making the sheep model a better comparison with humans than a rat model (46).

The importance of glucocorticoids in the control of energy homeostasis is exemplified by Zucker rats, where adrenalectomy normalizes body weight and glucocorticoid replacement results in increased weight gain (70, 71). The

role of glucocorticoids is highlighted in POMC knockout mice when pituitary POMC expression is restored. This activates adrenal glucocorticoid production and development of obesity (72).

The GR marker region used in this study is close to the rat GR $1_7$ promoter region, where epigenetic changes have been found concomitant with changes in hippocampal GR expression associated with altered maternal care (69). Also, recently, glucocorticoid responsive elements have been identified proximal to this region that are active in the auto-regulation of GR expression in human lymphoblastoid cells (60). In the current study, there was a marked increase in the H3K9Ac of the *GR* gene promoter in the hypothalami from fetuses exposed to maternal undernutrition. This histone modification would suggest open chromatin in the GR promoter region (69) and, together with observed hypomethylation of the GR promoter marker, would predict that *GR* gene expression is up-regulated. This change should be faithfully replicated during mitosis (69). Indeed, in this study, an increase in hypothalamic *GR* gene expression was associated with these observed epigenetic changes.

Because glucocorticoids can regulate both POMC and NPY, it is difficult to speculate on the implications of increased GR expression in the hypothalamus, because these neuropeptides have opposing effects on energy balance. It is well known that glucocorticoids decrease *POMC* gene expression in the pituitary, and therefore, it might be predicted that decreased POMC expression in the hypothalamus could up-regulate food intake (41, 73). However, there are tissue specific promoter regions in the *POMC* gene. It has been shown that 13 kb of *5′ POMC* gene flanking sequence are required for appropriate spatial and temporal expression in the hypothalamus (74), and recently, hypothalamic specific control regions have been well defined within this region (58, 59).

Therefore, it may not be surprising that glucocorticoids have been shown to up-regulate *POMC* gene expression in the hypothalamus as demonstrated in adrenalectomised rats, where the loss of glucocorticoids results in a decrease in POMC expression and this effect is completely reversed by replacement of physiological doses of glucocorticoid (75). POMC mRNA levels are also positively regulated by glucocorticoids in the rat ARC, in that adrenalectomy produced a marked decrease in POMC mRNA, and this was reversed by treatment with dexamethasone (76). Similarly, glucocorticoids up-regulate ACTH production in rodent hypothalamic arcuate neurons (77). Therefore, this evidence would predict glucocorticoid up-regulation of POMC expression, which would subsequently lead to a lean phenotype (72). In comparison, it is known that increased glucocorticoid signaling in NPY neurons would up-regulate NPY in animals given a high caloric diet (78).

In summary, pre- and periconceptional maternal undernutrition are associated with changes in late gestation in fetal hypothalamic genes involved in feeding regulatory networks. This suggests that minimizing risk of programming effects in humans needs to consider interventions before conception. The epigenetic modification of the genes is suggested by the H3K9Ac associated with *POMC* and *GR* genes and the hypomethylation of *POMC* and *GR* gene markers in the fetal hypothalamic neurons. Given that POMC and GR are centrally involved in modulating food intake, this potential for long-term programming could have implications for hypothalamic regulation of energy balance in adult offspring.

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